Applied Catalysis B: Environmental 342 (2024) 123404

Contents lists available at ScienceDirect

Applied Catalysis B: Environmental

journal homepage: www.elsevier.com/locate/apcatb

Exploring new galaxies: Perspectives on the discovery of novel PET-degrading enzymes

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ARTICLE INFO

Keywords:
PET recycling
Protein engineering
Enzymatic degradation
Microbial dark matter
AlphaFold
High-throughput screening

ABSTRACT

Polyethylene terephthalate (PET) is a widely used polyester due to its beneficial material properties and low cost. However, PET contributes significantly to the growing problem of plastic waste pollution. Enzymatic PET recycling has emerged as a promising alternative to conventional mechanical and chemical recycling methods. While many PET hydrolases – belonging to the α/β-hydrolase fold superfamily – have been discovered, the wild-type enzymes obtained from natural sources are not optimal for industrial conditions and need to be optimized through rational design or directed evolution to improve their efficiency and stability. This Perspective summarizes case studies of engineered PET hydrolases and proposes a workflow that tightly integrates a variety of *in silico* and high-throughput approaches for biochemical and structural characterization to accelerate the discovery of PET-degrading enzymes, also with novel structural scaffolds. These biocatalysts could be candidates for developing further innovative plastic recycling techniques.

1. Introduction

Polyethylene terephthalate is the most widely produced polyester utilized in packaging, clothing, and other materials, with 82 million tonnes of PET produced annually [1,2]. This is due to its excellent mechanical, thermal, and chemical properties, ease of chemical synthesis and polymer manufacturing, low cost of production, resistance to moisture, chemicals, and UV light, and many other factors [3]. The overuse of PET has resulted in a significant increase in plastic waste production globally. This mounting waste poses a daunting challenge for waste management facilities and often leads to the mismanagement of plastic waste, ranging from littering and leakage of plastic waste from

Abbreviations: 2-HEMHET4, 2-hydroxyethyl(MHET)4; 2PET, BHET-like analog of 2-HE(MHET)3; AI, artificial intelligence; ASR, ancestral sequence reconstruction; BEST-TROSY, a specific transverse relaxation optimized spectroscopy technique; BHET, bis(2-hydroxyethyl) terephthalate; CAR, carboxylic acid reductase; cryo-EM, cryogenic electron microscopy; CSP, chemical shift perturbation; EC number, Enzyme Commission number; EG, ethylene glycol; HEMT, 1-(2-hydroxyethyl) 4-methyl terephthalate; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence spectroscopy; HOTP, 2-hydroxyterephthalate; IMG/M, Integrated Microbial Genomes and Microbiomes database; IsPETase, Ideonella sakaiensis PETase; LCC, leaf compost cutinase; MHET, mono hydroxyethyl terephthalate; MHETA, 4-[ethyl(2-hydroxyethyl)carbamoyl]benzoic acid; MHETase, MHET hydrolase; MX, macromolecular crystallography; NADP, nicotinamide adenine dinucleotide phosphate; NCBI, National Center for Biotechnology Information; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PDB, Protein Data Bank; PES-H1, polyester hydrolase 1; PET, polyethylene terephthalate; PETase, PET hydrolase; PHL7, polyester hydrolase 7; pLDDT, predicted local distance difference test; pNP, para-nitrophenyl ester; PRE, paramagnetic relaxation enhancement; pTM, template modeling score; RDC, residual dipolar coupling; RF, RoseTTAFold; Tg, glass transition temperature; TIM barrel, triose-phosphate isomerase barrel; TPA, terephthalic acid.

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https://doi.org/10.1016/j.apcatb.2023.123404
Received 27 April 2023; Received in revised form 10 July 2023; Accepted 16 October 2023
Available online 23 October 2023

landfills to uncontrolled dumping into land and marine ecosystems, ultimately contributing to environmental pollution and adverse effects on human health [4]. For example, the formation of micro- and nano-plastics from PET waste, in particular, has been linked to various ecological and health hazards, highlighting the urgent need for sustainable plastic waste management strategies [5,6]. While global awareness of the plastic crisis has accelerated further development of existing mechanical and chemical plastic recycling techniques, biotechnological plastic recycling has recently emerged as a promising alternative for industrial applications [7–12]. The last group of enzyme-based recycling methods have numerous potential advantages over the former two options, including higher selectivity and efficiency in breaking down complex polymer structures, lower energy consumption and environmental impact, and the ability to recycle a wider range of plastic types.

PET is a polymer composed of ethylene glycol (EG) and terephthalic acid (TPA) repeating units linked by ester bonds (Fig. 1). Therefore, its complete depolymerization is biochemically possible and was demonstrated for the first time in 2005 by a cutinase named TfH that was isolated from *Thermobifida fusca* [13]. Since then, numerous PET hydrolases have been discovered and engineered to provide a more in-depth understanding of enzymatic polyester hydrolysis as well as to improve their catalytic efficiency to meet the requirements for industrial use [14]. As enzymes are too large to permeate insoluble polymer substrates, enzymatic PET hydrolysis is typically viewed as a surface erosion or interfacial biocatalytic process that can only occur at the liquid-solid interface [14,15]. In this context, increasing the accessibility of surface ester bonds to the catalytic center of enzymes has emerged as a major goal for the scientific communities. PET exhibits a semicrystalline structure characterized by both ordered (crystalline) and disordered (amorphous) regions. When the percent crystallinity of a PET sample exceeds 20%, the enzymatic degradation rate is extremely low, as is the case with most post-consumer PET waste, such as textile fibers and beverage bottles [10,16–21]. This is due to the highly rigid structure of crystalline regions unless the polymer is melted at temperatures of up to 260 °C [22,23]. By contrast, the amorphous regions can become mobile to interact with the biocatalysts when the glass transition temperature (T_g) is approached [16,17]. PET has a T_g of 65–71 °C in the absence of moisture [24,25]. In comparison, under the biocatalytic degradation condition, the water-plasticized PET surface layer can have a very low T_g of around 40 °C, allowing for degradation at ambient temperature [26].

Yoshida *et al.* reported in 2016 that the bacterium *Ideonella sakaiensis* can secrete a mesophilic PETase, allowing it to depolymerize amorphous PET and assimilate its monomers to support bacterial growth at 30 C [28]. Nonetheless, increasing the reaction temperature will dramatically increase the chain mobility of amorphous PET, thereby enhancing its degradability by enzymes [29–31]. These insights make the discovery of novel thermophilic enzymes – as well as thermostabilizing known PET hydrolases – an important trend in this active research field [32–35]. For industrial use, the generally preferred PET hydrolases are the ones with long-term stability and outstanding activity, catalyzing depolymerization of amorphized PET faster than it will recrystallize in the temperature range of 70–75 °C [14,16,32].

Experimental techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, complemented by bioinformatics analyses and molecular modeling, provided pieces of the puzzle to determine the structural basis for thermostability, substrate binding, and catalysis [34,36–39]. Understanding the determinants of these individual properties has led to the generation of PET hydrolase variants with improved activity and thermostability using targeted mutagenesis of rationally identified hot spots or directed evolution [32,35,36,40].

In this Perspective, we propose a workflow integrating *in silico* approaches, such as bioinformatics, molecular modeling, and machine learning (Fig. 2), with high-throughput biochemical and in-depth structural characterization (Fig. 3) to aid discovery of novel PET-hydrolyzing enzymes and we summarize case studies of PET hydrolases which have been already improved using a broad range of protein engineering methods ranging from rational design with computational tools to directed evolution. This Perspective thus aims to expand the available knowledge space and to define new avenues for

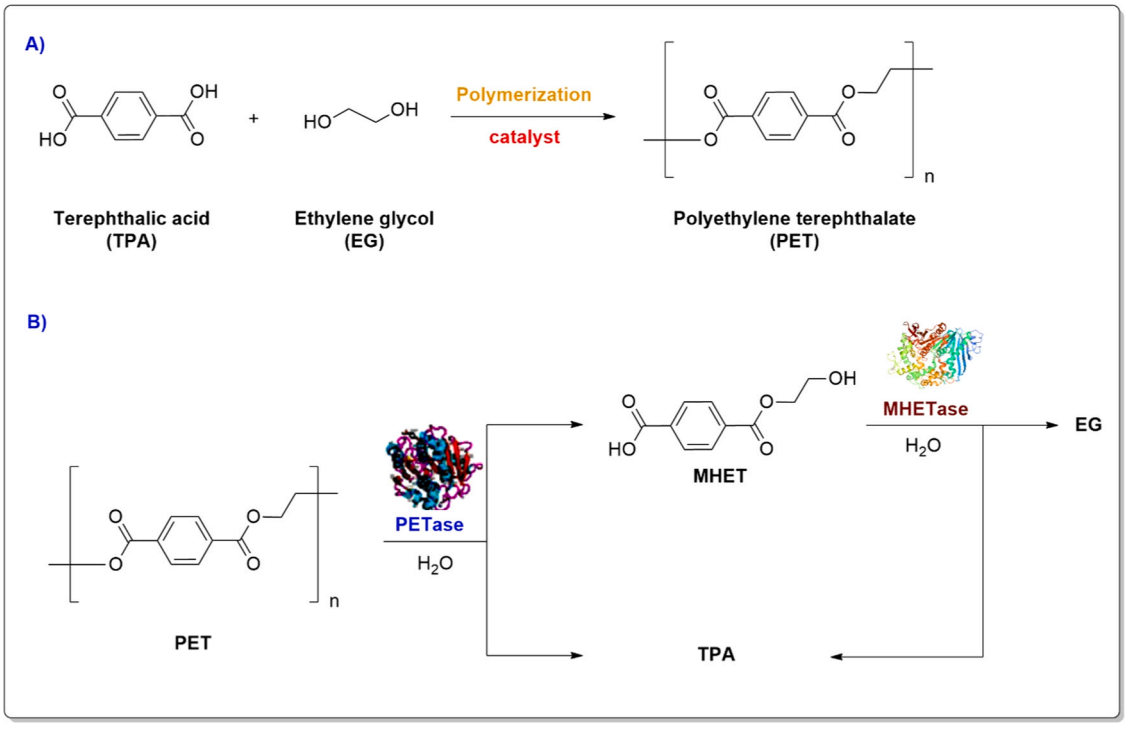

Fig. 1. : A: Scheme of the chemical step-growth polymerization to make PET from its monomers, TPA and EG. B: Scheme of the enzymatic depolymerization of PET by a PETase into shorter PET oligomers, and penultimate intermediates BHET and MHET, which can be degraded into monomers (usable for virgin PET synthesis) by a PETase with MHET-hydrolysing activity or a specialized MHETase, such as the *I. sakaiensis* MHETase or TfCa from *T. fusca* [27].

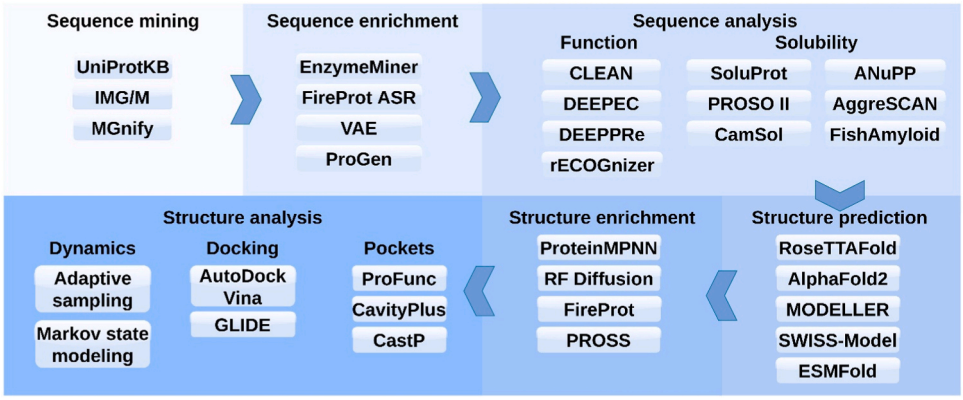

Fig. 2. : Proposed *in silico* workflow involves: (i) mining sequences from extremophilic organisms and plastic-polluted environments, (ii) sequence enrichment, (iii) sequence analysis and filtering, (iv) structure prediction, (v) structure enrichment, and (vi) structural analysis and prioritization. The figure also lists some of the available software tools for individual tasks.

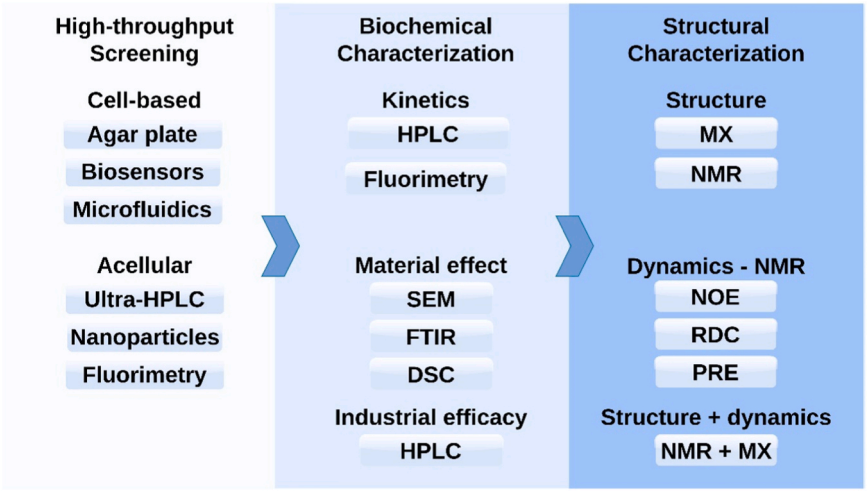

Fig. 3. : High-throughput screening of PET-degrading enzymes can be achieved using cell-based methods such as agar plate PET-degradation assays, biosensor assays, microfluidic techniques, or acellular assays. Detailed biochemical characterization of PET-degrading enzymes involves methods quantifying PET degradation efficiency, which can be divided into methods quantifying the released degradation products and methods investigating the modification of solid polymer material properties. The structural determinants of effective PET degradation can be explored using various methods of MX, NMR, or their combinations.

biotechnological solutions for PET recycling.

2. Discovery and characterization of novel PET-degrading enzymes

The main economic challenge of biotechnology-based PET recycling systems compared to the production of "virgin" PET from fossil fuels or using non-enzymatic traditional recycling methods has yet to be overcome. Due to their structural and functional similarities, PETases identified so far within the lipases and cutinases subfamilies have insufficient activity for an industrial process and are unlikely to provide new information to guide protein engineering. Furthermore, improving PET hydrolases for industrial PET degradation is complicated by various factors such as different expression systems, experimental temperatures and pH values, and the polymer properties of pretreated PET materials [14]. While the performance of enzymatic PET degradation is frequently optimized in academic labs using high buffer concentrations or adding detergents, the industrial feasibility of these engineered hydrolases should be re-evaluated in the most chemical-free process environments [34,41-43].

We thus propose expanding the sequence-structure-function information space also beyond the α/β-hydrolase fold superfamily using an *in silico* discovery-design workflow (Fig. 2) which may lead to economically viable enzymes with desired properties. Verifying proposed hits by testing critical activity and stability parameters in a high-throughput and replicable way will narrow down this space and make it accessible to the broad community of researchers.

Enzymes capable of depolymerizing PET and other plastics with hydrolyzable backbones have already been isolated from nature, wild-type PET hydrolases such as LCC, *Is*PETase, and PES-H1, which we describe in the Case Studies below, as well as many others [44–47]. These biocatalysts present only a fraction of what can be discovered by sequencing plastics-polluted environmental samples, terrestrial and marine metagenomes, plastic-eating worms, and the human microbiome [48–53]. Moreover, most of these enzymes have evolved to work under mesophilic conditions, not meeting the requirements for rapid depolymerization at industrial conditions [33,54,55]. Therefore, improved catalysis can be achieved by discovering proteins from extremophilic organisms, which display stability and activity at increased temperatures, or by redesigning mesophilic proteins using *in silico* stabilization and solubilization tools.

The discovery and engineering of PET hydrolases are not limited to the α/β-hydrolase fold superfamily [56]. A study by Erickson *et al.* demonstrated that enzymes with different folds could hydrolyze PET at temperatures ranging from 30 ℃ to 70 ℃ [56]. A sequence profile based on 17 known PET hydrolases was used for searching the NCBI

non-redundant database and thermophilic metagenomes. The authors prioritized the proteins from host organisms with optimum growth temperatures over 50 ℃ or sequences with high homology with thermostable protein sequences from the metagenome datasets. Of the 74 sequences selected for experimental characterization, 51 were successfully produced, and 31 displayed significant PET hydrolysis activities. Many identified proteins significantly diverged from the α/β-hydrolase fold superfamily, e.g., by having an accessory lid, carbohydrate-binding domains, or lacking half of the core domain [56].

Enzyme scaffolds such as the TIM barrel, α,β plaits, and others, can be functionalized for novel reactions using bioinformatics, molecular modeling, and machine learning-aided structural optimization, given the protein-ligand interaction knowledge [57–63]. The resulting enzymes can be further optimized using directed evolution, rational design, and novel machine learning-based strategies [35,64–66]. In this workflow (Figs. 2, 3), we illustrate the process of promising sequence discovery, enrichment, filtering, and refinement of candidate PET-degrading enzymes, as well as their high-throughput and detailed biochemical characterization and structural characterization. A similar strategy can also be applied to the discovery and design of enzymes degrading other plastics. Exploring new folds and engineering other types of catalytic machinery than the hydrolase one is important, especially since most plastics are non-hydrolyzable [67].

2.1. *In silico discovery of novel PET hydrolases*

Metagenomic databases storing the genes obtained by sequencing environmental, plant, animal, and human metagenome samples became a significant source for discovering novel enzymes. These sequences can provide a valuable starting point for further optimization of these biocatalysts by rational design or directed evolution [48,56,68]. Specifically, the proteins from extremophilic organisms and samples from PET-polluted environments may possess advantageous properties for efficient plastics degradation [28,69–71].

The most widely used database for sourcing candidate sequences is the UniProtKB database, which offers half a million human-verified sequences in SWISS-PROT and over 245 million automatically annotated proteins in TrEMBL [72]. In UniProtKB, there are currently over 69 million sequences annotated with hydrolase activity [73]. Moreover, the Integrated Microbial Genomes and Microbiomes database contains over 70 billion genes automatically assigned to their Pfam structural family and an EC number describing their catalytic activity, etc. [74]. These databases were used in 2017 to identify more than 500 putative PET hydrolases. Four hits were selected using the sequence comparison to known PET hydrolases and confirmed experimentally to be active on PET [48]. The metagenomic database MGnify contains more than a billion annotated microbiome sequences and aggregated BFD database sequence profiles of 65 million sequence clusters [75,76].

Sensitive sequence search tools based on hidden Markov chain models, such as HHBlits and HMMER, can be used to search the databases using alignment-based sequence profiles [77,78]. Sequence profiles can be further explored by PSI-BLAST and its alternatives such as CTX-BLAST, to be refined for sensitive homolog detection by incorporating retrieved candidate sequences into the profile for the next search iteration [79,80]. Furthermore, key residue profiles can be enhanced using sequences from specialized databases related to PET hydrolases. The PAZy database contains 187 enzymes with manual annotation of plastic-degrading activity toward 8 different plastics [81]. The PlasticDB contains almost 200 putative plastic-degrading proteins with experimental or AlphaFold2-predicted structures [82]. General databases of enzymes with detailed functional annotations, BRENDA or KEGG, can also be used for profile refinement [83–85].

2.2. *In silico analysis and improvement of novel PET hydrolases*

Enriching the set of candidate sequences can be done using several approaches. EnzymeMiner is a web server for mining the NCBI non-redundant sequence database using query sequences and residues essential for the enzymatic reaction [86,87]. The selection of enzymes in the resulting hit table can be based on the predicted solubility score, which predicts functional expression of the recombinant enzyme in *Escherichia coli*, as well as based on annotated extremophilic origin and other properties predicted or taken from databases. The selection of sequences can be balanced using a Cytoscape graph of the sequence similarity networks [88]. Another web tool (Fireprot^{ASR}) offers an alternative approach based on ancestral sequence reconstruction, which is a technique that generates evolutionary ancestors from a set of extant sequences using statistical approaches [89]. The first fully automated tool was comprehensively evaluated by reconstructing all 56 possible ancestral nodes based on 57 extant ene reductases [90,91]. The ancestral sequences exhibited higher expression levels, showed an average ΔT_m improvement of +9 ℃, and were both highly active and enantioselective [91].

The sequence and structure space of specific enzyme datasets can be learned by language models, variational autoencoders, and other generative machine-learning methods [92–94]. These methods differ in their functionality, e.g., variational autoencoders provide a visual representation of the sequence distribution, which may give insights into the molecular determinants of their properties. However, all generative models typically allow refining and enriching candidate sequences by generating *de novo* sequences following trends toward valuable properties. Several protein families have been explored this way, including soluble and functional variants of luciferases, malate dehydrogenases, chorismate mutases, lysosymes, and nanobodies with increased binding [95–99]. These promising results demonstrate that machine learning-based algorithms can learn intricate relationships in protein data and use them to generate functional protein variants. Moreover, some synthetically generated sequences shared very little sequence identity with known enzymes. For instance, the recently published large deep-learning model ProGen successfully generated functional lysozyme sequences with sequence identities lower than 40% to any known natural protein for two lysozyme families [99]. Structure-generating deep networks were also shown to "hallucinate" stable proteins with sequences unrelated to native proteins. This implies the tremendous potential that machine learning provides in significantly enriching the candidate sequence space [100,101].

Additional sequence analyses are typically required to prioritize sequences for more demanding structural and experimental studies. Function prediction can be made using machine learning-based web servers such as CLEAN, DeepEC, DEEPre, HECNet, and others [102–105]. For example, HECNet reported the accuracy of 91% sequence-based predicting of Enzyme Commission (EC) Numbers up to the fourth digit, which is equivalent to predicting the capability to hydrolyze PET [105]. Prediction of functional motifs, structural domains, and taxonomic classifications can be achieved using the rECOGnizer web tool trained on annotated data from UniProtKB and tested on metagenomic datasets [106]. A few sequence-based predictors of protein stability have also been published recently, although it remains to be seen whether their performance will be maintained in future independent testing [107,108]. Despite the high accuracy of the above-mentioned tools for E.C. number prediction, it is not nearly as reliable to assign a function to a metagenomic sequence significantly different from known sequences encoding enzymes that catalyze the associated reaction. The functional annotation methods should thus be compared with each other for consensus, should take into account the assessed sequence dis/similarity, and should ideally be supplemented with additional analyses, namely structure prediction and analysis of the putative catalytic site to be able to accommodate PET depolymerization, and to possess necessary residues, using pocket detection methods and molecular docking. Moreover, tools specifically trained for prediction of plastic-degrading capability have been developed recently, and their usage could be beneficial for correct function annotation [109].

Solubility (referring to the expression of soluble enzyme candidates in a heterologous host such as *E. coli*) is a crucial property for protein production and yield. Low aggregation propensity is crucial for industrially used enzymes, as illustrated by the engineering of LCC^{ICCG} under process conditions, which we are discussing in the Case Studies section. One possibility to account for this challenge is to use predictors trained on solubility data, such as SoluProt, PROSO II, CamSol, and others, although they demonstrated only a modest overall accuracy of 54–58% on an independent dataset [110–112]. Another possibility is to use predictors trained on aggregation data, e.g., ANuPP, AggreSCAN, and FishAmyloid, showing accuracies in the range of 64–83% for binary soluble/insoluble classification of proteins expressed in *E. coli* [113–115]. Overall, solubility and aggregation propensity prediction tools do not guarantee a precise prediction of soluble sequences but provide a powerful way to prioritize and enrich the dataset in sequences of potentially soluble enzymes [110]. Eliminating likely poorly soluble and aggregating sequences can also be done using transmembrane region detection tools, such as DeepTMHMM and TOPCONS2, achieving overall accuracy of 98% and 92%, respectively [116,117]. Moreover, structures that are unlikely to fold into desired domains can be detected using the secondary structure content predicting tools PSI-PRED or SABLE, which both predict the secondary structure type with an accuracy of 77% [118,119]. Although the presented methods reach high accuracy using diverse test datasets, soluble production of metagenome-derived proteins in a heterologous host can be very challenging. For successful expression, it is advised not to rely on a single prediction but to select a set of sequences based on the consensus of several techniques utilizing different approaches and assessing various aspects contributing to the soluble yield mentioned above. Moreover, the likelihood of soluble protein expression can be increased by the optimization of production strains and vectors, co-expression with chaperones, fusion with solubilization tags, and other techniques [120,121].

Structure prediction is the fourth step of the proposed workflow (Fig. 2). Recent machine learning-based methods, such as AlphaFold2, RoseTTAFold, and ESMFold, provide accurate structural predictions along with confidence metrics: (i) the overall confidence of the entire predicted structure pTM, (ii) region-specific confidence pLDDT, and (iii) Predicted Aligned Error, the confidence of relative position of two protein regions to each other, describing inter-domain position confidence [93,122,123]. Based on the results of the CASP14 evaluations, AlphaFold2 and RoseTTAFold are similarly accurate. Also, ESMFold provides an order of magnitude-faster predictions completed within seconds. On the CAMEO test set, AlphaFold2, RoseTTAFold, and ESMFold were reported to have average pTMs of 0.88, 0.82, and 0.83, respectively [93]. These methods allow the structure generation of large datasets. It is worth mentioning that traditional homology modeling-based methods, such as MODELLER or SWISS-MODEL, may still perform better for sequences with close homology to ones with known experimental structures [124,125].

The sequences of the candidate structure set can also be enriched using machine learning tools. Graph neural network-based ProteinMPNN has successfully rescued *de novo* sequences designed using AlphaFold2 and Rosetta frameworks that failed to express previously. The sequences of complex cyclic homo-oligomers redesigned using ProteinMPNN greatly enhanced soluble protein yields and exhibited well-defined structures [126].

Structures of putative PET-degrading enzymes can be further prioritized via detailed visual inspection and molecular modeling. Substrate binding pockets can be identified and prioritized using the ProFUNC, CavityPlus, and CastP algorithms [127–129]. These pockets can then be characterized by molecular docking, a method for filtering out structures which are incapable of PET endo-cleavage. Tools such as AutoDock Vina or Glide can be used for high-throughput screening of binding capabilities of the structures in the candidate set, as well as reveal novel binding and catalytic structural motifs [130,131]. Molecular dynamics simulations have a broad range of uses in PET-degrading enzymes study and engineering. The dynamics of the catalytic site geometry have been studied using MD simulations to explain the activity of the PET-degrading enzymes *Is*PETase and LCC and to describe the effects of mutations on their activity [36,132–134]. The thermal stability of PET-degrading enzymes can be improved using MD simulations by discovering flexible regions in their structure and making them more rigid via directed mutagenesis, which was applied to the PET hydrolase TfCut2 [135]. MD simulations also offer information about substrate binding, product dissociation, and product inhibition, which can be used to design the activity of PET hydrolases, as was previously demonstrated [34,39].

Structures can also be optimized for stability using the popular web tools FireProt and PROSS, which integrate phylogenetic analysis and force field-based predictions of the effect of a mutation [136,137]. Finally, the *de novo* design of sequences folding into suitable structural motifs is possible via the RoseTTAFold Diffusion algorithm [101]. The RF Diffusion tool, tightly integrated with the aforementioned ProteinMPNN, was evaluated by designing proteins around 25 diverse structural motifs. These structures ranged from viral epitopes and binder proteins to ferredoxin and carbonic anhydrase active sites. Experimental characterization showed that most of the designed sequences fold into the desired highly thermostable structures [101].

2.3. Methodology for biochemical characterization and screening of novel enzymes

Enzymatic PET hydrolysis is an interfacial catalysis process that occurs at the solid-liquid interface [14]. As such, there are two main categories of characterization methods for enzymatic PET degradation performance: those that monitor the modification of the residual polymer substrate, and those that monitor the release of water-soluble degradation products.

For bulky PET substrate forms such as films or granules, weight loss determination is the most straightforward way to quantify degradation performance. However, PET materials contain a low concentration of low-molecular-weight oligomers, e.g., cyclic trimers, formed as byproducts of polymer synthesis [138,139]. Numerous ester hydrolases that do not depolymerize PET, e.g., CalB, TfCa, can readily hydrolyze these oligomers, indicating that weight losses of less than 1% are not necessarily attributable to PET depolymerization [27,140]. Furthermore, weight loss determination is a one-sample-at-a-time process and, therefore, unsuitable for screening large mutant or metagenomic libraries. Alternatively, the change in polymer properties resulting from enzymatic PET hydrolysis can be verified using various other methods such as scanning electron microscopy, Fourier-transform infrared spectroscopy, NMR, and differential scanning calorimetry [16,29,31,141,142]. However, these methods can only provide qualitative property change information and can hardly quantify the polymer degradation rate.

To enable both rapid mechanistic characterization and high-throughput screening methods for novel PET hydrolases or variants, nano-sized PET model compounds such as ultra-thin films or nanoparticles can be utilized [26]. PET nanoparticles can be produced by dissolving PET in, e.g., hexafluoro-2-propanol, followed by precipitation into water [20]. The mean particle size of nanoparticles prepared using this method is typically around 100 ± 50 nm, and they have significantly larger specific surface area and lower molecular weight than those of the pristine PET before preparation, making them more easily hydrolyzable by enzymes [33,34,143]. The turbidity decrease of a PET nanoparticle suspension can be easily detected spectrophotometrically (e.g., at 600 nm) as a result of enzymatic hydrolysis, thus making it a suitable method for kinetic analysis [20,34,144,145]. When the nanoparticles are embedded in agar plates, clear zones can form around the bacterial colonies expressing active PETases following incubation at specific conditions [48]. This way, agar plate assays with very high

throughput can be developed to screen millions of clones for novel or more active PETases each week [33,141,144,146]. To characterize polyester hydrolase activities, similar assays have been developed with PET-related oligomers or commercially available polyester-polyurethane dispersions [147–150]. The use of PET nanoparticles can speed up the screening process to identify promising PETases or variants, the hydrolytic activity of which should be investigated further using real-world waste PET materials.

Terephthalate and its oligo-esters with EG such as MHET and BHET, are water-soluble UV-absorbing hydrolysis products of PET. Thus, they can be easily monitored in a spectrophotometer at 240 nm or by HPLC [141,151]. The latter has so far become the most widely used method to quantify PET hydrolase activities. Recently, ultra-HPLC has demonstrated its utility in the rapid characterization of a large number of PETase variants [36,56]. The release of terephthalate can lower the pH value of the reaction mixture. Thus, colorimetric assays based on pH indicators have also been developed to monitor the enzymatic hydrolysis of PET or BHET, but this method is less reliable because determining pH change with mutant libraries expressed in microtiter plates is rather error-prone [147,148].

TPA can be easily converted into the fluorophore 2-hydroxyterephthalate by a reaction with hydroxyl radicals formed by, e.g., a Fenton-like reaction [152,153]. This serves as the foundation for the most prevalent fluorescent assay to detect PET hydrolysis products. Using PET nanoparticles as the substrate, PETase activity in crude *E. coli* lysates can be readily distinguished by fluorimetric detection [154]. Since other major hydrolysis products, such as MHET and BHET, cannot be easily determined by this method, modifications have recently been published, such as the introduction of MHETase for rapid conversion of MHET to TPA prior to the Fenton-like reaction or the use of a synthetic HOTP-based di-ester as the substrate [155,156]. These modifications have merely increased the robustness and reliability of this method for high-throughput screening. In addition to TPA, Gimeno-Pérez *et al.* recently described a fluorescence assay for detecting the PET hydrolysis product EG [157]. This method uses a ketoreductase to oxidize EG, MHET, or BHET to the corresponding aldehydes in the presence of the cofactor $NADP^+$. The *Clostridium kluyveri* diaphorase then re-oxidizes NADPH to $NADP^+$ and catalyzes the reduction of resazurin to resorufin, which is highly fluorescent (580 nm) when excited in the visible light range (550 nm). As the signals derived from MHET and BHET will interfere with those of EG in certain concentration ranges, which are quite common of an enzymatic PET hydrolysate, this method still has its limitations for accurate quantification, making it at present less attractive than TPA-based fluorescence assays.

Additional fluorescence-based assays that do not target the hydrolysis products have been reported. For instance, various synthetic fluorogenic substrates were injected into droplets containing, e.g., bacterial cells able to express PETase variants [158,159]. After incubation for cell growth and the hydrolysis reaction, the droplets are sorted on the basis of their fluorescence. The use of fluorogenic surrogate substrates that bear little resemblance to PET is a drawback of this method. These will also enable the detection of false positive hits, such as other promiscuous esterases that lack depolymerizing activity and must be rescreened using substrates with a high molecular weight. Furthermore, an intriguing method involves trapping the fluorogenic substrate fluorescein dilaurate in PET films prepared in a microtiter plate well [149]. The hydrolysis of PET by enzymes liberates the fluorogenic substrate, which is subsequently hydrolyzed to produce fluorescence. Although this method does not directly target PET hydrolysis products, it does target the deformation of bulky polymers.

Biosensors for the reaction product TPA could enable ultrahigh-throughput screening of PETase activities. Pardo *et al.* identified the *Acinetobacter baylyi* ADP1 muconate transporter MucK as a terephthalate transporter using the terephthalate biosensor TphR from *Comamonas testosteroni* [160]. The authors propose that their biosensor could also be useful for high-throughput screening of large libraries for PET hydrolase activities producing terephthalate. Li *et al.* engineered mutants of XylS from *Pseudomonas putida* that can bind phthalate and TPA specifically and then constructed an *E. coli* whole-cell biosensor that can detect phthalate or TPA at 10 μmol/L [161]. While the utility of this biosensor has been validated by screening a phthalatase mutant library and identifying significantly improved mutants, it has yet to be evaluated for PET hydrolase screening. Later, Bayer *et al.* described the first TPA biosensor combining carboxylic acid reductase (CAR) and luciferase LuxAB from *Mycobacterium marinum*, which was validated by PET hydrolase activities [162]. LuxAB from *Photorhabdus luminescens* was introduced into *E. coli* to enable the detection of structurally diverse aldehydes, such as those obtained by TPA after CAR-catalyzed conversion. The semi-quantitative detection of TPA as a product of enzymatic PET hydrolysis correlates well with those determined by the standard HPLC method. More recently, Dierkes *et al.* developed a fluorescent biosensor based on the TPA-transporting and -metabolizing *Comamonas thiooxidans* strain S23 [163]. The most promising deletion mutant lacking the catabolic genes involved in TPA degradation demonstrated up to 10,000-fold greater sensitivity for detecting TPA in the nanomolar range than the wild-type strain. Biosensor strains with varying sensitivities would be useful for applications ranging from metagenome screening (which requires high sensitivity) to protein engineering for enhancing already-effective PET hydrolases (which need to be less sensitive). None of the aforementioned four methods were directly validated with the actual screening of real libraries, indicating that they all require further optimization for various experimental purposes in future practical applications.

2.4. Methodology for structural characterization of novel enzymes

Atomic-resolution structures are fundamentally contributing to the understanding of biomolecular mechanisms, and PET hydrolases are not an exception. Typically, macromolecular X-ray crystallography is the method of choice to determine structures of smaller globular proteins such as hydrolases, as seen in the Protein Data Bank, in which all 70 currently deposited PET hydrolase entries are crystal structures obtained by MX. However, several scenarios may require alternative methods for the structural characterization of PET hydrolases; for example, a target is difficult to crystallize, crystals do not diffract to high resolution, or co-structures with bound substrates are not possible [164,165]. In this section, we detail the benefits of MX for the characterization of PET hydrolases. We critically discuss structural modeling and docking approaches but also evaluate additional potential avenues, e.g., electron microscopy or studies employing NMR that may be applicable for a comprehensive structural characterization of enzymatic PET degradation.

Despite continuous reports of enzymes capable of degrading synthetic polymers over two decades, the discovery of a naturally existing enzyme cascade in the bacterium *I. sakaiensis*, has led to a significant expansion of research activities on PET hydrolysis [28,166–168]. *I. sakaiensis* employs two enzymes, *Is*PETase (hydrolyzing PET to mainly MHET) and MHETase (hydrolyzing MHET to TPA and EG). In particular, with the discovery of *I. sakaiensis* and its two main PET hydrolases, MX has kept pace with the structural characterization of newly discovered plastic-degrading enzymes, starting with structures of *Is*PETase and MHETase in their substrate-bound states shortly after their discovery [39,169]. In light of the high structural similarity of hydrolases in general, homology modeling servers and the recently emerging AI-based computed structural models have enabled the prediction of novel hydrolase structures with high confidence [93,122–124,170]. This has reduced the relevance of hydrolase apo-structures that are now rather determined for the validation and development of mutational strategies [32,39]. In contrast, substrate-bound co-crystal structures of PET hydrolases are of markedly higher relevance since they cannot be reliably predicted with docking models and yield valuable insights into the enzyme-substrate encounter [29,171]. The difficulty of obtaining these

co-crystal structures is mirrored by the relatively small fraction of around 13 substrate-bound PET hydrolases among the 70 MX-derived structures deposited in the PDB (Table 1). Taking a closer look at these co-crystal structures, a catalytically competent interaction of the substrate (mimetic) with the active site residues and aligning well with the oxyanion hole is rather the exception than a rule. The only experimental strategies available to obtain co-structures are catalytic triad mutants with an authentic ester substrate or wild-type enzymes with a non-hydrolyzable amide analog, the latter originally introduced for this system with the first MHETase co-structure [34,27,169,171,172]. While each strategy has its advantages and disadvantages, it is not predictable which one will lead to a successful co-crystal structure and whether other experimental parameters should be considered. For example, Han *et al.* discovered in their first apo-structure of *I. sakaiensis* PETase that an arginine residue was blocking the active site within the packing of the crystal lattice and mutating this residue to a glycine enabled them to obtain co-structures with HEMT as a substrate [39]. One other point may be that since the substrate is highly repetitive, other substrate binding sites just in the vicinity of the active site may be of high relevance, as shown recently [34].

One other strategy to obtain structural approximations of substrate binding is molecular docking. This option is enabled through a large set of different software tools that produce comprehensive docking models relatively quickly and are a useful alternative in the absence of co-crystal structures [175]. PET hydrolases have been intensively studied by molecular docking, as we have compiled in Supplementary Table 1.

Of these docking results, although with different software and protocols, the authors all claimed that they chose poses with the lowest binding energy, and some were further energy minimized with MD simulations. There are different kinds of ligands used in the docking analysis, smaller ligands, such as MHET, or larger ligands, such as 2PET, 2-HE(MHET)3, 2-HE(MHET)4, PET, and PET tetramers, etc. (Table 1). The catalytic serine of these docking results is close to the reaction-related ester bond, and it should be correct for the catalytic mechanism elucidation. Still, the accuracy for the binding of larger ligands to residues and positions far away from the cavity is less convincing. Despite the usefulness of PET hydrolase docking models, we recommend that overinterpretation of these results should be avoided unless the docking models have been experimentally validated [29,39].

Table 1
Co-crystal structures of PET-degrading hydrolases deposited in the Protein Data Bank.

Hydrolase	Substrate	PDB code	Experimental strategy	Ref.
*Is*PETase Arg103Gly/ Ser131Ala	pNP	5XH2	Active site mutant Ser131Ala is inactive. The Arg103Gly mutation prevents Arg103 from sterically blocking the active site during crystallization	[39]
	HEMT	5XH3	Active site mutant Ser131Ala is inactive. The Arg103Gly mutation prevents Arg103 from sterically blocking the active site during crystallization	[39]
MHETase	MHETA	6QGA	MHETA is an amide and not hydrolysable	[169]
MHETase	BHET	6JTT	Serendipitous discovery, the strategy is not conceivable	[173]
TfCa	MHETA	7W1J	MHETA is an amide and not hydrolysable	[27]
TfCa Glu319Leu	BHET, MHET	7W1I	Active site mutant Glu319Leu is inactive	[27]
TfCa Glu319Leu	BHET	7W1L	Active site mutant Glu319Leu is inactive	[27]
PES-H1	MHETA	7W6C, 7W6O, 7W6Q	MHETA is an amide and not hydrolysable	[34]
PES-H2	BHET	7W66	BHET is not bound productively	[34]
LCC^{ICCG}	MHET	7VVE	Active site mutant Ser165Ala is inactive	[34]
Ple629	MHETA	7VPB	MHETA is an amide and not hydrolysable	[172]
TfCut	MHET	7XTV	Active site mutant Ser130Ala is inactive	[174]
TfCut	MHET	7XTT	Active site mutant Ser130Ala is inactive	[174]
*Is*PETase	MHET	7XTT	Active site mutant Ser131Ala is inactive	[174]

NMR spectroscopy detects the proximity and neighborhood of thousands of atomic nuclei across the protein under investigation [176]. It is a powerful technique to analyze different samples, including chemicals, biomolecules, and the whole body, from different organisms, organs, tissues, cells, biofluids, etc. [177]. In protein structure determination, NMR spectroscopy can be regarded as a complementary technique since it sits on the interface between structural characterization by crystallography, cryo-electron microscopy, and other biophysical techniques [178]. Protein structural information can be obtained by NMR spectroscopy implementing different approaches that include nuclear Overhauser effect, residual dipolar coupling, or paramagnetic relaxation enhancement [177]. Structure determination for proteins larger than 50 kDa is particularly challenging using solution-state protein NMR spectroscopy, due to rapid nuclear relaxation caused by the slow molecular tumbling which leads to low detection sensitivity, resulting in shortage of obtained information about distances and local conformation. However, the molecular weight range can be expanded to higher than 50 kDa by the introduction of specific methyl labeling with $^{13}CH_3$ groups and deuteration, unfortunately, information about the backbone conformation cannot be obtained since this approach is limited to methyl groups, which in turn excludes any *de novo* structure determination [178–181]. Despite the limitations of protein NMR spectroscopy, it can be used to study proteins for the elucidation of dynamics, conformations, and atomic-resolution structures, the characterization of protein-protein and protein-ligand interactions, as well as other interactions with proteins [182,183].

Only a few examples of PET hydrolases are available in the literature, which includes the utilization of NMR spectroscopy. Lippens and co-workers used various NMR probes, including histidine side chain, methyl group, and backbone amide resonances, to investigate the interaction of enzyme LCC and its four mutations with MHET [184]. In that regard, a series of $^{1}H–^{13}C$ HMQC was recorded to obtain $^{1}H–^{13}C$ histidine mapping, a series of $^{1}H–^{13}C$ HSQC was recorded to get $^{1}H–^{13}C$ methyl mapping, and a series of $^{1}H–^{15}N$ BEST-TROSY were recorded to achieve $^{1}H/^{15}N$ mapping. They also carried out a CSP analysis for calcium metal by recording a series of $^{1}H–^{15}N$ TROSY spectra at 800 MHz. Their findings were in agreement with previous studies based on crystallographic data [185,186]. Lippens and co-workers implemented high-temperature NMR spectroscopy, recording $^{1}H–^{15}N$ TROSY spectra, to evaluate the thermostability of LCC by identifying regions being unfolded upon heating.

Courtade and co-workers implemented NMR spectroscopy to study the kinetics of the PET hydrolase cutinase FsC isolated from *Fusarium solani pisi* at different enzyme concentrations and various pH values [187,188]. Also, they mapped the interaction between BHET and FsC by identifying substrate-binding residues on FsC. Time-resolved NMR experiments were utilized to monitor product release in real time.

Pepermans and co-workers probed the backbone dynamics of FsC by NMR to monitor internal motions on different time scales. In their study, they compared protein conformation elucidated by X-ray crystallography with those obtained in solution and elucidated by NMR [189,190]. In the crystal structure, the hydrophobic binding site is exposed, suggesting that FsC has a rather open conformation. However, backbone dynamics of FsC probed by NMR in solution revealed the presence of

interconversion between closed and open conformations, similar to in true lipases. They concluded that the crystal structure fortuitously represents the active conformation which is one of the multiple conformations present in the solution.

Cryo-EM has not yet been used to study the structures of PET hydrolases. However, an authentic substrate-bound state, e.g., to nanoparticles, films, or thin fibers, would be particularly intriguing. In this context, the small size of the hydrolases remains the primary obstacle. With a molecular mass of about 30 kDa far below the detection limit of the electron microscopes (approximately 100 kDa), attempts to obtain EM-structures would suffer from severe difficulties in locating these hydrolases. Even when bound to an amorphous PET nanoparticle and thus localizable, the PET moiety would not contribute to reconstructing the structures that depend on averaging.

3. State of the art and case studies

In 2005, Müller *et al*. demonstrated that enzymatic degradation of low-crystallinity PET is possible [13,191,192]. They discovered that the cutinase TfH from *T. fusca* can erode up to 17 μm of a thin PET film after three weeks of incubation at 55 ℃ [13]. In the following 18 years, many highly homologous cutinases to TfH from different *Thermobifida* species have been identified and engineered for enhanced PET depolymerization at elevated temperatures up to 70 ℃ [145,193–198]. Moreover, other thermostable PET hydrolyzing enzymes, such as the leaf-compost cutinase and PES-H1 (also known as PHL7) were discovered using metagenomic approaches, and HiC was discovered in the thermophilic fungus *Thermomyces insolens* (formerly *Humicola insolens*) [34,54,140,199]. These enzymes can efficiently degrade amorphous PET at 70 ℃ or higher and thus have a promising potential industrial use.

In this section, we aim to illustrate the discovery and engineering of three archetypal enzymes systematically explored in the domain of PET degradation: 1) LCC engineered into LCC^{ICCG}, the benchmark PET hydrolase in engineering studies and industry; 2) *Is*PETase, which provided structural insights for improving the activity of other PET hydrolases and which was improved for higher stability by introducing features from its thermostable counterparts; and 3) PES-H1, a metagenome-derived PET hydrolase engineered using multiple substrate binding

Table 2
Examples of successful engineering of enzymes used for degradation of PET.

Template	Mutations	Targeted property	Achieved improvement	Engineering principle	Ref.
LCC	Phe243Ile	Thermostability, catalytic activity	$\Delta T_m = -3.1$ ℃, $T_m = 81.6$ ℃, 1.30-fold increased activity at 65 °C	Structure-guided saturation mutagenesis	[32]
LCC	Phe243Trp	Thermostability, catalytic activity	$\Delta T_m = +1.4$ ℃, $T_m = 86.1$ ℃, 1.19-fold increased activity at 65C	Structure-guided saturation mutagenesis	[32]
LCC	Tyr127Gly	Thermostability, catalytic activity	$\Delta T_m = +2.3$ ℃, $T_m = 87.0$ ℃, 1.33-fold decreased activity at 65 ℃	Structure-guided saturation mutagenesis	[32]
LCC	Asp238Cys/Ser283Cys	Thermostability, catalytic activity	$\Delta T_m = +9.8$ ℃, $T_m = 94.5$ ℃, 1.28-fold decreased activity at 65 ℃	Structure-guided disulfide bridge introduction via directed mutagenesis	[32]
LCC	LCC^{ICCG}: Phe243I/ Asp238Cys/ Ser283Cys/Tyr127Gly	Thermostability, catalytic activity	$\Delta T_m = +9.3$ ℃, $T_m = 94.0$ ℃, 1.01-fold increased activity at 65 ℃	Structure-guided saturation mutagenesis, disulfide bridge introduction	[32]
LCC	LCC^{WCCG}: Phe243Trp/ Asp238Cys/ Ser283Cys/Tyr127Gly	Thermostability, catalytic activity	$\Delta T_m = +13.3$ ℃, $T_m = 98.0$ ℃, 1.08-fold decreased activity at 65 ℃	Structure-guided saturation mutagenesis, disulfide bridge introduction	[32]
*Is*PETase	Ser238Phe/Trp159His	Catalytic activity	$\Delta T_m = +9.7$ ℃, $T_m = 58.5$ ℃, 1.15 fold improved activity at 30 ℃	Bioinformatics comparison with thermostable PET hydrolases, narrowing catalytic site via structure-guided site-directed mutagenesis	[18, 36]
*Is*PETase	ThermoPETase: Ser121Glu/ Asp186His/ Arg280Ala	Thermostability	$\Delta T_m = +8.8$ ℃, $T_m = 57.6$ ℃, 14-fold increased activity at 40 ℃	Bioinformatics comparison with thermostable PET hydrolases, hydrogen bonding network expanded via structure-guided site-directed mutagenesis	[40]
*Is*PETase	DuraPETase: Ala214His/ Ile168Arg/ Trp159His/Ser188Gln/ Arg280Ala/Ala180Ile/ Gly165Ala/Gln119Tyr/ Leu17Phe/Thr140Asp	Thermostability	$\Delta T_m = +31.0$ ℃, $T_m = 77.0$ ℃, 300-fold increased activity at 37 ℃	GRAPE greedy optimization algorithm utilizing force-field stability evaluation & sequence consensus from 9 PET hydrolases, gene synthesis of resulting variants	[55]
*Is*PETase	DuraPETase/Asn233Lys	Thermostability, catalytic activity	$\Delta T_m = +38.4$ ℃, $T_m = 83.5$ ℃, 9.7-fold improved activity at 30 ℃, 115-fold increased activity at 40 ℃	Machine-learning, Salt bridge introduction via directed mutagenesis	[200]
*Is*PETase	HotPETase: ThermoPETase (Ser121Glu/Asp186His)/ Arg280Ala/Pro181Val/ Ser207Arg/Ser214Tyr/ Gln119Lys/Ser213Glu/ Arg90Thr/Gln182Met/ Asn212Lys/Arg224Leu/ Ser58Ala/Ser61Val/ Lys95Asn/Met154Gly/ Asn241Cys/ Lys252Met/Thr270Gln	Thermostability, catalytic activity	$\Delta T_m = +37.5$ ℃, $T_m = 82.5$ ℃, 3.4-fold increased activity at 65 ℃	Bioinformatics, structure analysis, and literature on PET hydrolases-guided identification of hot spots, directed evolution of hot spot regions	[35]
PES-H1	Arg204Cys/Ser250Cys	Thermostability, catalytic activity	$\Delta T_m = +6.4$ ℃, $T_m = 91.3$ ℃, no change in activity	Bioinformatics and structure-guided comparison with more stable PET hydrolases, disulfide bridge introduction via directed mutagenesis	[34]
PES-H1	Leu92Phe/Gln94Tyr	Thermostability, catalytic activity	$\Delta T_m = +1.8$ ℃, $T_m = 86.7$ ℃, 1.24-fold increased activity at 72C	Bioinformatics and structure-guided comparison with PET hydrolases, site-directed mutagenesis	[34]

modes discovered by molecular docking and dynamics, as well as knowledge from other PET hydrolases [28,32,54,168]. The progress made in the design of these three PET hydrolases is summarized in Table 2.

3.1. LCC

Leaf and branch compost cutinase is a thermostable protein discovered via a metagenomic approach [53]. It is composed of 259 amino acids and adopts the α/β-hydrolase fold [54]. However, LCC has a narrower, less flexible binding cleft containing the catalytic residues Ser165, Asp210, and His242. The catalytic cleft is primarily occupied by aromatic and hydrophobic amino acids. In contrast to another PET hydrolase discussed in the next section, *Is*PETase, Trp190 rotamerization is prevented in LCC by interaction with the adjacent residues His218 and Phe222. LCC contains one disulfide bridge between Cys275 and Cys292, and the elimination of this bond leads to the loss of its stability [54,201].

LCC has a melting point of 84.7 ℃ and an optimum temperature for PET degradation is approximately at 70 ℃ [32]. LCC is able to degrade 40% of low-crystallinity PET at 70 ℃ in 24 h, while *Is*PETase degrades only 1% at its optimum temperature of 30 ℃ [29]. LCC exhibits both thermodynamic and kinetic stability, as the unfolding rate of LCC is very slow when subjected to the chemical denaturant guanidinium hydrochloride and at elevated temperatures [201]. The T_{50} of LCC was measured to increase by at least 20 ℃ in the presence of PET, indicating stabilization of the active site by the substrate [201].

LCC's high stability and activity have led to its extensive protein engineering, especially as reported by Tournier *et al.* [32]. The authors performed a molecular docking to identify 11 hot spot positions and subjected them to saturation mutagenesis. In total, they evaluated 209 variants for improved activity. Variants with improved activity carried Phe243 mutations to Ile or Trp. The authors also derived a variant containing a second disulfide bridge by substituting positions 238 and 283 with Cys residues, where homologous PET hydrolases contain a calcium-binding site. Introducing this disulfide bridge increased T_m to 94.5 ℃ and rendered the mutant thermostable without the need for a high concentration of calcium ions [32,202]. Stability and activity-enhancing mutations were combined into several mutants, with the LCC^{ICCG} variant showing the best conversion level of 90% within 10.5 h, being able to degrade 200 g/L of thermo-mechanically amorphized PET into its monomers on the multitone scale (Table 2). These monomers were then recovered, purified and used to synthesize virgin PET, which has identical properties to petrochemical-derived PET, thereby closing the recycling loop efficiently. LCC^{ICCG} displayed the best productivity-to-enzyme cost ratio according to this study and is now being developed for economically viable industrial PET degradation by the Carbios company [32]. The full-scale industrial plant with a capacity for recycling 50,000 tonnes of PET/year is scheduled to start operation in 2025 (A. Marty, Carbios, personal communication). Quantum-mechanical studies have revealed that LCC^{ICCG} has similar reaction thermodynamics to *Is*PETase [38]. This has led to focus design efforts on engineering better PET absorption and catalysis at the optimal reaction temperature up to 72 ℃. This temperature range is high enough for the mobilization and accessibility of PET chains and below the point (75 °C) where fast temperature-promoted physical aging and re-crystallization of PET (within 3 h) hinders its degradation [14]. With recent progress in the development of machine learning, these methods were used for enhancing both thermostability and catalytic activity of PET hydrolases [55,200].

3.2. IsPETase

Another milestone in enzymatic PET degradation was achieved in 2016 when a PET hydrolase was discovered, which is secreted by the bacterium *Ideonella sakaiensis*. This enzyme, *Is*PETase, is able to degrade amorphous PET at ambient temperature such as 30℃ [18,28,39]. *Is*PETase is a 263 amino acid long protein that is homologous to lipases and cutinases [28]. Similar to these enzymes, it adopts the α/β-hydrolase fold (Fig. 4) [36]. *Is*PETase has an isoelectric point of 9.6, contrasting to other cutinases, which are neutral. The overall charged enzyme has a binding site composed of many hydrophobic, aromatic residues, which allow binding PET oligomers close to its catalytic triad of Ser160, Asp206, and His237. *Is*PETase's canonical "lipase box" motif Gly-X1-Ser-X2-Gly has variable positions around the catalytic serine occupied by Trp159, π-stacking with the substrate, and Met161. Moreover, substrate binding to the near-attack conformation of breaking of the ester bond is enhanced by Trp185 rotamerization, and the reaction tetrahedral intermediate is stabilized by the oxyanion hole formed by Met61 and Trp87. *Is*PETase's active-site cleft is broader than in its homologs. *Is*PETase's ability to degrade PET at an optimum temperature range of 30–40 ℃ was attributed to its high structural dynamics, which is kept in order by a unique second disulfide bridge between Cys203 and Cys239 [38]. *I. sakaiensis* also produces an MHETase, which can catalyze the hydrolysis of MHET, a PET degradation intermediate that can inhibit *Is*PETase, into TPA and EG, which can be assimilated by this bacterium for growth [28,168,169]. Thus, for PET recycling, coexpression of *Is*PETase with MHETase can mitigate substrate inhibition of *Is*PETase by the penultimate product MHET [40]. This dual enzyme system then yields recyclable monomers and enables the closure of the recycling loop [39,169]. The structure of *Is*PETase and the reaction mechanism of enzymatic PET degradation are shown in Fig. 4.

The thermostability of *Is*PETase, with a T_m of 46.8 ℃ was improved by introducing mutations inspired by residues present in thermostable PET hydrolases, yielding a T_m improvement of 9.7 ℃ [18,36]. *Is*PETase was also mutated into the ten-point variant DuraPETase using force field-based calculations and sequence consensus to other PET hydrolases [55]. DuraPETase was further improved by replacing its calcium-binding site with a salt bridge, yielding the most thermostable *Is*PETase variant with a T_m of 83.5 ℃ [200]. ThermoPETase, a structural comparison-based triple mutant of *Is*PETase, was further developed by the deep learning algorithm MutCompute to yield a thermostable mutant (FAST-PETase), which was superior to previously designed *Is*PETase variants for the degradation of semicrystalline PET [40,200]. Recently, directed evolution was used to generate HOTPETase, which was claimed to outperform the benchmark PET-degrading enzyme LCC^{ICCG} in semicrystalline PET powder at 65 ℃ over up to 48 h and optimal buffer conditions for each of the two enzymes [35].

3.3. PES-H1

Polyester hydrolase 1 (PES-H1) is a 258 amino acid-long protein derived from a compost metagenome library [34,201]. This enzyme is highly efficient in depolymerizing PET at high temperatures, surpassing the efficacy of wild-type LCC [29,34,54]. PES-H1 has a highly similar structure to LCC and *Is*PETase, containing the catalytic triad composed of Ser130, Asp176, and His208. A flexible Trp155 residue is π-stacking with aromatic moieties of PET and its degradation products, similarly to *Is*PETase, and contains a disulfide bridge (Cys241/Cys256), which is conserved among known PET hydrolases [34,203]. PES-H1 lacks a calcium-binding site, which is present in many PET hydrolases, and contains positively charged residues at homologous positions [34]. PES-H2, a natural variant containing four mutations compared to PES-H1 (Glu1Ala/ Phe209Leu/Asn232Asp/Ala254Ser), was also derived from the compost metagenome library [34,191]. The mutation Leu209 located in the catalytic site is a recognized hot spot for enhancing PET degradation activity in LCC^{WCCG}, LCC^{ICCG}, and PET hydrolase TfCut2 [196,204]. Interestingly, all substitutions prepared by the saturation mutagenesis of Leu209 reduced catalytic activity, while many have slightly increased T_m [34].

Pfaff *et al.* improved PES-H1 by creating variants using site-directed mutagenesis based on previously identified mutations [34]. A disulfide bridge (Cys204/Cys250) was introduced, resulting in a T_m increase of

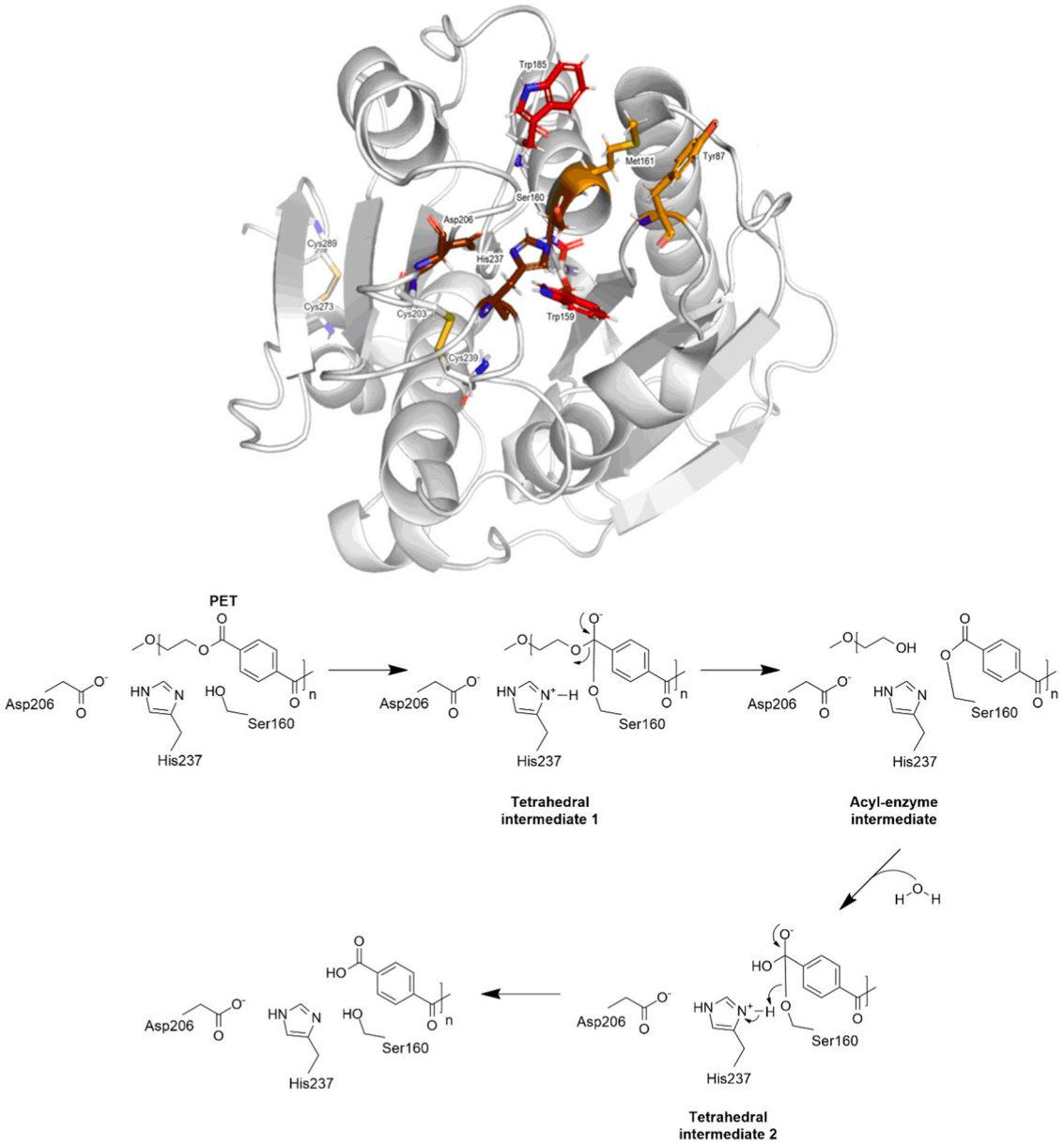

Fig. 4. Top: Structure of *Is*PETase with its catalytic site (Ser160, Asp206, His237) highlighted in dark brown, oxyanion hole formed by Tyr87 and Met161 highlighted in orange, key substrate-binding residues Trp158 and Trp185 highlighted in red, and two disulfide bridges of Cys203/Cys239 and Cys273/Cys289 are highlighted in yellow. Bottom: Reaction mechanism of enzymatic PET degradation: The reaction is initiated via a nucleophilic attack of the catalytic serine on the ester carbonyl carbon atom. The resulting tetrahedral intermediate is stabilized by hydrogen bonding by the catalytic histidine, catalytic aspartic acid, and the oxyanion hole followed by breakdown of the tetrahedral intermediate into an acyl-enzyme intermediate. A water molecule is subsequently activated by the catalytic histidine/aspartate pair. The resulting hydroxyl ion attacks the acyl-enzyme intermediate's carbonyl carbon, forming a second tetrahedral intermediate. This is followed by the ultimate deacylation step, resulting in product release and restoration of the catalytic amino acids.

6.4 ℃. Moreover, PES-H1 was further stabilized by mutations identified in a DuraPETase stabilization study [55]. The resulting Leu92-Phe/Gln94Tyr double mutant was reported to be superior to LCC^{ICCG}, achieving a 2.4-fold higher degradation of melting-quenched PET powder obtained from real-world waste bottles within 24 h at 72 ℃. This suggests that the PES-H1 Leu92Phe/Gln94Tyr double mutant might be a more efficient PET hydrolase than LCC^{ICCG}. However, the experiment was conducted in 1 M phosphate buffer at pH 8.0, which is optimal for PES-H1. LCC^{ICCG} may thus still be economically more feasible since its lower content of superficial charged residues leads to its stability at significantly lower buffer concentrations [34,191].

4. Conclusions

In the last eighteen years, there has been a significant increase in the discovery and characterization of PET hydrolases. The initial discovery and description of the PET hydrolase TfH was groundbreaking, as it showed that enzymatic PET degradation is possible. This provided a promising alternative to traditional mechanical and chemical methods [13,28,32,54]. Since then, numerous PET hydrolases from various carboxylesterase subclasses of the α/β-hydrolase fold superfamily have been isolated from various sources, including environmental samples and metagenomic libraries. These PET hydrolases have been extensively studied, using biochemical, structural, and computational approaches, to understand their properties, structures, and reaction mechanisms.

Collected knowledge provided the basis for protein engineering

efforts to improve their activity and stability, with remarkable successes, such as the engineered enzyme LCC^{ICCG}, which is capable of degrading PET with over 90% efficiency on a multiton scale [32]. However, the economic feasibility of using these highly stable and active enzymes for PET recycling remains a challenge and further improvement of PET degrading enzymes is desirable. Considering the significant amount of resources invested into this research direction and the number of involved research groups, recent progress is somehow limited due to replicated engineering of only a limited number of structural scaffolds. Diversification of these structural scaffolds by advanced enzyme mining of genomic and metagenomics databases, structural bioinformatics analyses, and AI-powered designs, will provide evolvable starting points for future engineering efforts.

Despite significant progress in engineering highly active PET hydrolases and gaining a better understanding of the structural and mechanistic aspects of enzymatic PET degradation, the economic feasibility of industrial-scale biocatalytic recycling is still limited by factors such as the high cost of amorphizing post-consumer bottles or fibers [2,191]. Therefore, to compete with traditional non-enzymatic recycling methods or virgin polymer production from fossil fuels, biotechnological PET recycling needs to improve its market competitiveness by reducing the cost of mechanical pretreatment. Although all known PET hydrolases have similar highly conserved structures (they all belong to the α/β-hydrolase fold superfamily), it is still unclear how one may overcome limitations in their catalytic ability to degrade crystalline PET and how to efficiently address inhibitory degradation intermediates [14,205]. Moreover, different research groups have used inconsistent experimental conditions to compare different PET hydrolases, making the selection of suitable enzyme candidates from academic studies for industrial-scale applications challenging [14,56,191,206].

Our Perspective suggests exploration of PET-degrading enzymes focusing not only on members of the α/β-hydrolase fold superfamily, but also diverse structural folds, such as the TIM barrel, α,β plaits, and others [56–58]. Erickson *et al.* have already identified novel PET hydrolases with folds and catalytic sites significantly different from PET hydrolases known thus far [56]. This study demonstrates the possibility of discovering enzymes with divergent structures, which are capable of PET degradation, through *in silico* metagenomic mining. The outlined *in silico* enzyme discovery and engineering pipeline can be used to extend the sequence and structural space of PET-degrading enzymes. Numerous bioinformatics and machine-learning tools, high-throughput screening techniques, and robust assays for detailed biochemical and structural characterization provide an arsenal of methods to explore more distant structural “galaxies” and develop PET-degrading enzymes with improved properties. The advantage of these general approaches is that they can be re-applied also for the identification and design of other enzymes, e.g., novel MHETases for suppression of product inhibition, depolymerases that can work on crystalline PET, or proteins for recycling and degradation of other plastic materials.

CRediT authorship contribution statement

Jan Mican: Writing – original draft, Writing – review & editing, Visualization, Project administration; **Da’san M. M. Jaradat:** Writing – original draft, Writing – review & editing, Visualization; **Weidong Liu** Writing – original draft, Writing – review & editing, Visualization; **Gert Weber:** Writing – original draft, Writing – review & editing, Visualization**; Stanislav Mazurenko:** Writing – original draft, Writing – review & editing; **Uwe T. Bornscheuer:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition; **Jiri Damborsky:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition; **Ren Wei:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition; **David Bednar:** Conceptualization, Project administration, Writing – review & editing, Supervision.

Declaration of Competing Interest

None declared.

Data availability

No data was used for the research described in the article.

Acknowledgement

Brno Ph.D. Talent Scholarship holder JM acknowledges funding from the Brno City Municipality. This publication is based upon work from COST Action COZYME of JD supported by European Cooperation in Science and Technology (grant number CA21162). This work has been accomplished during the sabbatical leave granted to DMMJ from Al-Balqa Applied University in the academic year 2022/2023. GW acknowledges funding received within the frame of the "Helmholtz Sustainability Challenge" project FINEST. The authors UTB and RW gratefully acknowledge the financial support received from the European Union’s Horizon 2020 research and innovation program (MIX-UP, grant number 870294; upPE-T, grant number 953214). The authors JD and DB acknowledge the financial support received from the Czech Ministry of Education (TEAMING, grant number CZ.02.1.01/0.0/0.0/17_043/0009632; ESFRI RECETOX, grant number LM2023069; ESFRI ELIXIR CZ, grant number LM2023055), the Technology Agency of the Czech Republic (grant number FW03010208), and European Union’s Horizon 2020 (CETOCOEN Excellence, grant number 857560).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.apcatb.2023.123404.

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